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ORIGINAL RESEARCH

Evaluation of Antimicrobial Photodynamic Therapy against *Streptococcus mutans* Biofilm *in situ*

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ABSTRACT

Aim: This study investigated the effect of antimicrobial photodynamic therapy (aPDT) over *Streptococcus mutans* biofilm.

Materials and methods: Eighteen (n = 18) patients were selected and one palatine device with dental blocks was used. The biofilm was treated by curcumin and Photogem® with a LED and the effect was analyzed by CFU/ml.

Results: Although, statistical analysis showed significant reductions for aPDT mainly with Photogem® (p = 0.02), these were low.

Conclusion: The results suggest a low antimicrobial effect of aPDT over *S. mutans* biofilm. Some parameters used need to be improved.

Clinical Significance: This technique can be a promising in Dentistry.

Keywords: Biofilm, Curcumin, Dental caries, Photodynamic therapy, Randomized clinical trial, *Streptococcus mutans*.

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INTRODUCTION

Dental caries is an infectious-contagious disease that has a chronic multifactorial pattern. It has been shown that the microorganisms are essential for the development of dental caries, despite only their presence is not enough. Hygiene, alimentary habits and saliva composition, among others, influence the metabolism of bacteria, modulating caries activity.[1]

The oral cavity is colonized by a diverse community of bacteria. Most of them are present as complex aggregate known as biofilm on the surface of the teeth, restorative materials, orthodontics appliances, dental implants and others.[2,3]

Different species of *Streptococcus* (*sobrinus, mutans* and *sanguinis*), and *Lactobacillus acidophilus* are some of the most common bacteria present in the oral environment.[4] These bacteria secrete organic acids as a product of the metabolism of fermentable carbohydrates. This process leads to the demineralization of tooth hard-tissue or cavitation in advanced stages.[5] Management of early carious lesions includes preventive approaches, such as biofilm removal, through different dental home care, professional placement of sealants, topical fluoride applications and the use of antimicrobial agents.[6,7]

One alternative has been the antimicrobial photodynamic therapy (aPDT)[8-10] defined as eradication of target microorganisms by reactive oxygen species.[4,11-15] Antimicrobial photodynamic therapy is effective for the treatment and prevention of dental caries, because it is

capable of sensitizing bacterial cells, demonstrating successful antimicrobial activity.[16-18]

The inactivation of bacteria by aPDT is based on that a specific photosensitizer (PS) can accumulate in or pass through of over the cytoplasmic membrane, which is the critical target for inducing irreversible damage to bacteria after irradiation.[19] However, the efficacy is dependent of several factors, such as the wavelength and its interaction with the photosensitizer, the power output, the length of pre-irradiation and irradiation times, the beam diameter, the operation mode of the light source (continuous or pulsed) and the convergence of the beam (focused or unfocused).[20,21]

Additionally, when aPDT is applied over biofilms, the effectiveness may be compromise, as well as the absorption reduction of the PS and light within their structure.[22-26]

The bacterial killing has been described as a result of chemical and phototoxic reactions, in which PS absorbs photons and induces the formation of free radicals and reactive oxygen species (ROS) reacting with nonspecific targets, such as cell membranes and proteins, which lead to bacterial destruction.[27,28] Compared to other antimicrobial agents, aPDT does not cause side effects.[15]

An ideal PS should be nontoxic and display local toxicity only after activation by illumination, high target specificity, and little likelihood of leading to the development of resistance by microorganisms.[29-31]

Many reports have shown the interaction between light sources and different PSs that absorb red wavelength, such as Photogem®, methylene blue (MB), toluidine blue ortho (TBO) and malachite green (MG).[32-35] Additionally, some research has also shown that blue light is an interesting option, because it can be used in combination with other photosensitizers, such as rose bengal (RB), eosin (EOS) and erythrosine (ERI).[36,37]

More recently, curcumin has been cited as potential photosensitizer.[15,38,39] Curcumin has a variety of traditional pharmaceutical applications for diseases, including wounds, liver diseases, microbial effects, and inflamed joints.[34]

Curcumin has proved nontoxic in a number of cell culture and whole animal studies. It has a rather broad absorption peak in the range of 300 to 500 nm (maximum 430 nm) and exerts potent phototoxic effects in micromolar amounts. Therefore, curcumin has potential as a PS for treatment of localized superficial infections in the mouth.[40] Additionally, it has economical advantages considering its low cost, simple manipulation and great effectiveness.[41]

Thus, the purpose of this study was to evaluate the effect of aPDT using Photogem® and curcumin, on *in situ* *Streptococcus mutans* biofilm. The null hypothesis was that there was no difference between the photosensitizers used.

MATERIALS AND METHODS

Subject's Selection

This study was submitted and approved by Ethics Committee in Human Research (Federal University of São Carlos—UFSCar, São Paulo, Brazil, Protocol: 194/2010).

Eighteen (n = 18) healthy volunteers were selected (13 women, 5 men), 18 years of age. Uncontrolled systemic diseases, smokers, alcoholics, who made continued use of antimicrobial rinses, and patients with edentulous prosthesis were not admitted.

They were informed about the study and signed a consent form previously the beginning of the study and answered a questionnaire about general and oral health.

Experimental Design

Eighteen volunteers wore palatal devices containing eight bovine enamel/dentin blocks. At the end of the clinical phase, the blocks were randomly allocated into one of the following treatments: Group I (Control): no photosensitizer and no light (PS-L-); Group II: curcumin and no light (PS+L-); Group III: curcumin and light (PS + L +) and Group IV: Photogem® and light (PS + L+).

Specimens Preparation

Seventy-two freshly bovine incisor teeth (n = 72) were used. The teeth were stored in 0.01% (v/v) thymol solution at 4°C (± 1°C). One hundred and forty-four (n = 144) enamel/dentin blocks (5 × 5 × 2 mm) were obtained using a water-cooled Isomet slow-speed diamond saw (Isomet 1000; Buehler, Lake Bluff, IL, USA). The teeth were autoclaved at 121°C during 15 minutes.

For each subject, an acrylic palatal device similar to Hawley type was fabricated in which eight cavities (5 × 5 × 2 mm) were prepared on the left and right sides (Fig. 1). One block was attached with wax in each cavity to allow biofilm accumulation (Fig. 1).

During the lead-in period (4 days) and throughout the clinical phases, the volunteers brushed their teeth with a fluoridated dentifrice [Sorriso Super Refrescante, Colgate-Palmolive, São Paulo, São Paulo, Brazil]. Also, they received oral and written instructions to wear the appliances at all times, including at night. They removed the appliances only during meals, when consuming acid drinks and performing oral hygiene. When removed, the devices were kept moist in plastic boxes to keep the bacterial biofilm viable.[42]

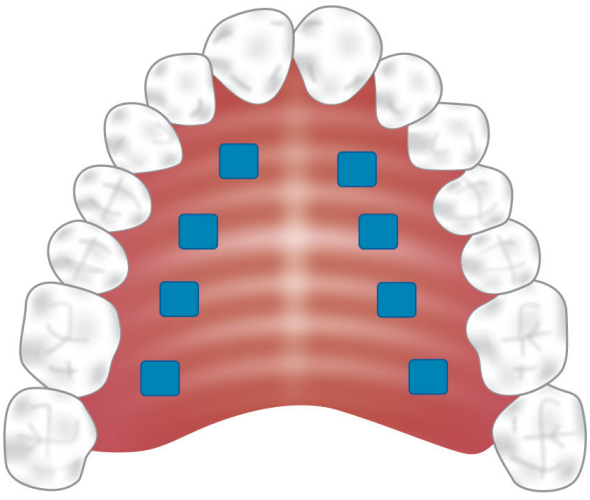


Fig. 1: Design of acrylic palatal device with bovine enamel/dentin blocks (5 × 5 × 2 mm). Modified from Lima JPM et al 2009.[32]

The cariogenic challenge was provided by a 2% sucrose solution onto all the bovine blocks, 4 times a day to stimulate the biofilm formation.[43] This solution remaining on the blocks during 10 minutes before replace acrylic palatal device.

After, a 5 minutes waiting time was standardized to allow diffusion of the sucrose on the biofilm. Brushing with the dentifrice was performed three times a day, after mealtimes when the volunteers habitually carried out their oral hygiene. After tooth brushing, they were asked to rinse their mouth with water before the use of acrylic palatal device.

The appliances were brushed extraorally, except for the block area, and volunteers were asked to brush carefully, to avoid disturbing the biofilm.

After 4 days, they returned to the dental clinic, the blocks were removed and the treatments were performed.

Photosensitizers (PSs)

The specimens were immersed for 5 minutes in a solution containing either Photogem® at 1000 µg/ml (Photogem, Moscow, Russia) or curcumin at 1500 µg/ml (PDT Pharma Indústria e Comércio de Produtos Farmacêuticos Ltda—EPP, Cravinhos, São Paulo, Brazil). These were prepared with distilled water and stored in a dark room until the beginning of the experiments.

Light-Source

Irradiation was performed with two light devices based on LED (Laboratory and Technological Support—LAT, Optical Group, Physics Institute of São Carlos, São Paulo, Brazil, Fig. 2). One device emits blue (450 ± 5 nm) and the another one red light (630 ± 5 nm), under a power density of 0.764 and 0.381 W/cm², respectively.

The irradiated area on the block was 0.250 cm² during 1 or 2 minutes with blue or red light, respectively, corresponding to an energy density of 45 J/cm² (Table 1) (Fig. 2).

The formula used to calculate the doses was as follows:

$$\text{Dose} = \frac{P(W) \times t(s)}{A(cm^2)}$$

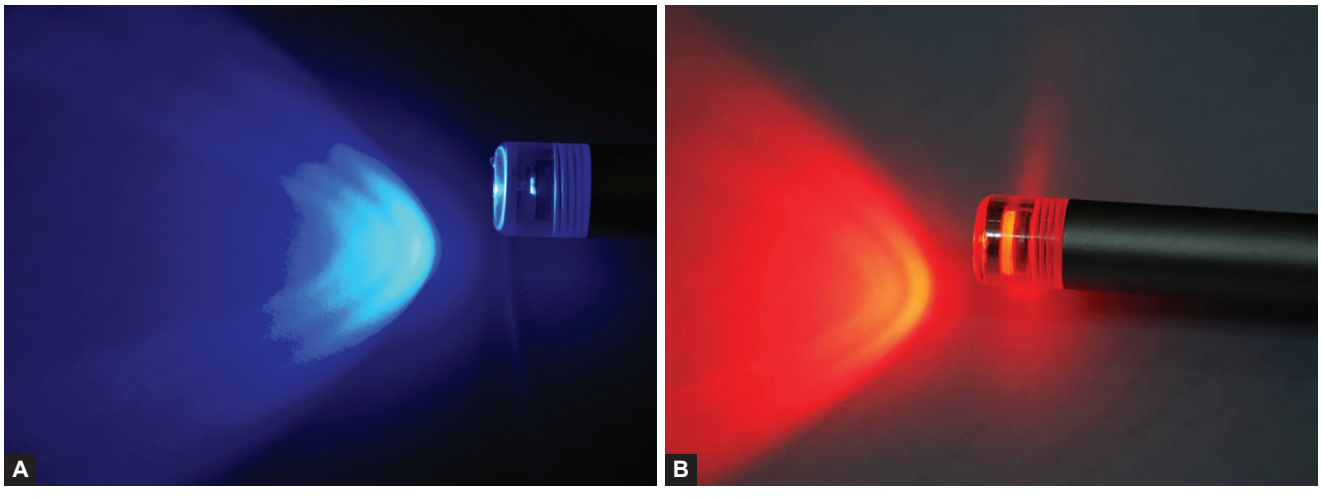
P = power output, t = irradiation time and A = dental block area.

Antimicrobial Photodynamic Therapy

Antimicrobial photodynamic therapy aPDT was performed using a random distribution of the blocks into the treatments. The groups PS+ L– and PS + L+ maintained contact with 500 µL of PSs during 5 minutes in the dark. Control group (PS-L-) received an equal

Table 1: Parameters of the light units used

Wavelength (λ) (nm)	*450 nm (± 5 nm)*	*630 nm (± 5 nm)*
Focal area (cm^2)	0.250 cm^2	0.250 cm^2
Power density (W/cm^2)	0.764 W/cm^2	0.381 W/cm^2
Power (W)	0.191 W	0.095 W
Irradiation time (minute)	1 minute	2 minutes
Fluency (J/cm^2)	45,84 J/cm^2	45,72 J/cm^2



Figs 2A and B: Light devices based on LED: (A) blue LED at 450 nm and (B) red LED at 630 nm

volume of sterile 0.9% NaCl solution during the same period. Irradiated groups PS + L+ during 1 (45J/cm^2) or 2 minutes (45J/cm^2) for curcumin and Photogem®, respectively, while the group PS + L– was submitted to a 6 minutes waiting period to simulate the irradiation conditions.

Microbiological Analyses

Bovine blocks were collected after aPDT and transferred separately to a sterile falcon tube (EO Sterile Q' TY:50 PCS, China) containing 1.5 ml of phosphate buffered saline (PBS) and three glass beads, and then subjected to shaking for 60 seconds. The serial decimal dilutions (1:10, 1:100, 1:1,000, 1:10,000, 1:100,000 and 1:1,000,000) were made (Fig. 3). The suspensions were transferred (0.1 ml) under vortex mixing immediately before the transfer to the petri dish.

To assess bacteria viability, samples were plated in triplicate on mitis salivarius agar, bacitracin and sucrose (MSA agar plus 0.2 units of bacitracin/ml) to determine total mutans streptococci.[44] After serial decimal dilution, the bacteria suspension was plated on petri dishes using drop plate technique (30 µl, Fig. 4). The plates were incubated under microaerophilic conditions for 48 hours at 37°C (± 1°C). After incubation, the total number of CFU was determined (Fig. 4).

STATISTICAL ANALYSIS

The variable log reduction was analyzed. Shapiro-Wilks test was used to test the data normality. The log-reduction results were calculated by subtraction of the initial from the final values of CFU after being transformed by Log_{10}. These data were analyzed using one-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni test at significance level of 5% ($p \leq 0.05$). The software Statistica for Windows Release 7 (Statsoft Inc., Tulsa, Oklahoma, USA) was used.

RESULTS

The data were reported as log reductions. Survival fraction was calculated by counting the colonies (PS + L+) and dividing by the number of colonies from the control group (PS – L–). The effects of aPDT (Fig. 5 and Table 2).

Antimicrobial photodynamic therapy showed a significant reduction in the number of CFU ml^{-1}, as (Figs 5 and 6), respectively.

However, differences among mean survival fractions for the different groups were quite low. The highest reduction was showed using Photogem.®

The results showed (Fig. 5) the inhibition of the biofilm after different treatments: Group II only curcumin, group III: curcumin + light and group IV: Photogem® + light compared to the control group (Group I). The barycenter is the center of mass of the data as shown in Figure 5. Above the axis 0 (zero), which represents the lack of aPDT efficacy, it was possible verify a coincidence with the center of barycenter. The vertical axis of Fig. 5 shows the mean CFU/mL in log_{10} obtained from the control group and treated groups ($\Delta = log_{10}$ control – log_{10} specific).

The percentage reduction when comparing group I (control) with the other groups are shown in Table 2.

The association of curcumin or Photogem® + LED resulted in a significant decrease in the total viability of streptococci $p = 0.04$ and $p = 0.02$, respectively (Fig. 6).

Table 2: Mean values, standard deviation (normalized log_{10}) and reduction percentage of *S. mutans* biofilm for the groups evaluated

Group	*Mean values*	*Standard deviation*	*Reduction percentage of S. mutans biofilm*
Control	5.84	1.24	—
Curcumin	5.33	1.0	8
Curcumin + Light	4.96	1.22	15
Photogem® + Light	4.79	1.36	18

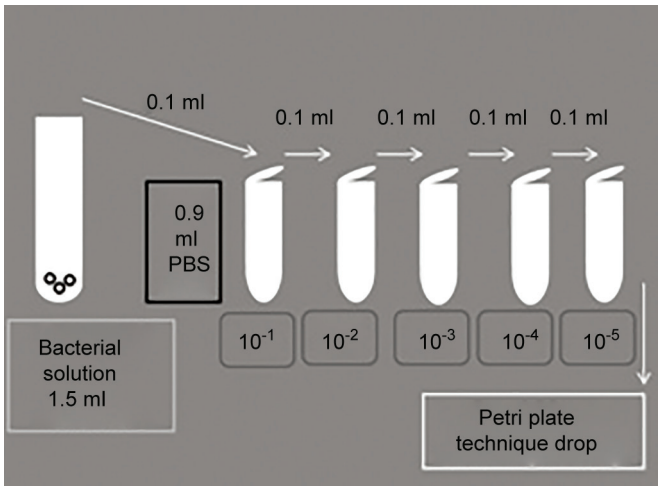


Fig. 3: Schematic drawing for serial dilutions

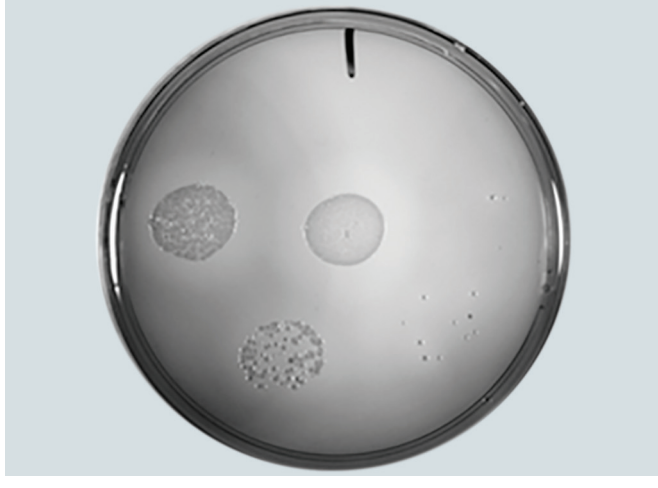


Fig. 4: The microflora (*S. mutans*) after incubation for 48 hours

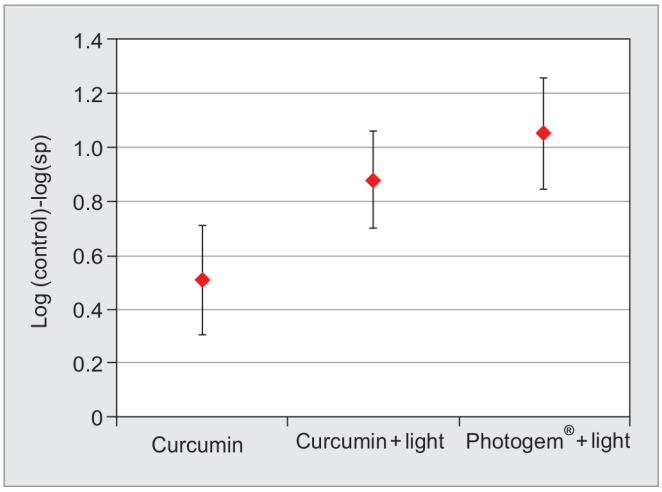


Fig. 5: Effect of the different treatments used: curcumin without light (PS + L–); curcumin (PS + L+) and Photogem® + light (PS + L+) on the *S. mutans* biofilm

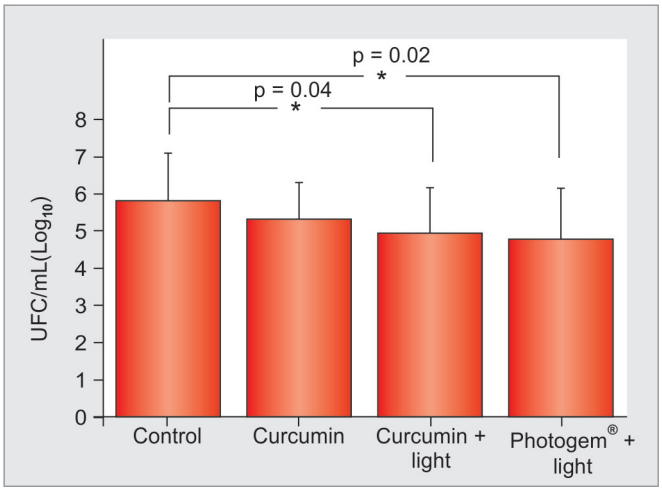


Fig. 6: Antibacterial effect for the control and experimental groups with curcumin, curcumin + light and Photogem® + light on the *S. mutans* biofilm

DISCUSSION

Management of dental caries is related to prescribing therapeutic regimens to individuals according to their risk levels and optimal conservative treatment decisions.[45] Based on this premise, the development of rapid, painless, atraumatic dental treatments to control cariogenic biofilms, including the use of antimicrobial agents, have been employed for dental disinfection.[46-48] Eradication of pathogens with a noninvasive method is an important issue for oral care and therapeutics.[47]

The current study focuses on the efficacy of aPDT using curcumin and Photogem® at 1500 and 1000 µ/ml irradiated with blue and red LED, respectively at the same doses to promote the killing of the biofilm.

Under the experimental conditions, based on statistical analysis, it was observed a significant reduction in the number of *S. mutans* when aPDT was used (Group III, p = 0.04 and Group IV, p = 0.02) comparing to control (group I). The greatest rate reduction was observed for group IV (Photogem®). However, the percentage reduction found was very low (15–18%). Also, it was not observed a significant reduction, when group II was evaluated showing a low dark cytotoxicity of this photosensitizer.

Curcumin has been extensively investigated for therapeutic applications due to its anti-inflammatory, antitumor, and antimicrobial effects.[49] Its antimicrobial effect is directly related to the combination or not with a visible light source.[50]

In this study, the reduction of bacterial viability was observed when curcumin was associated with blue LED, however, was very low. Likewise, the studies of Dahl et al[50] and Tønnesen et al[51] reported that the antibacterial activity of curcumin was greatly enhanced by light.

Williams et al noted 100% death of *S. mutans* in a planktonic suspension, using LED with TBO.[52] Neither TBO dye nor light alone showed a significant antibacterial effect under the experimental conditions used. These results and our findings highlight the need for dye-light conjugation to ensure the effectiveness of aPDT. The photodynamic effects of the dye and light were also confirmed by Giusti et al.[16] Photogem® and TBO activated by red light caused reduction of *L. acidophilus* and *S. mutans* in carious dentin.

The antimicrobial photodynamic action occurs due to production of highly reactive oxygen leading to death of microorganisms.[4] The bacteria are inactivated due to changes made mainly in its cytoplasmic membrane; however, reactions also occur with other components.[53]

Some studies have applied aPDT on cariogenic bacteria in the planktonic phase and not organized as a biofilm. This fact can explain the results obtained in this study, because oral biofilms are very organized structures that difficult the aPDT action. The reduced susceptibility may also be attributed to the reduced penetration or diffusion of photosensitizers.[54,55] It has been suggested, that water channels can carry solutes into or out of the depth of a biofilm, but they do not guarantee access to the interior of the cell clusters[52] whose diameter may range from 20 to 600 µm.[56] In addition, biofilm differ from planktonic (suspended) because they are surrounded by an extracellular polymeric matrix, which hinders its inactivation and also have different metabolic activity and gen expression.[57,58] To get the aPDT efficacy is necessary that PSs diffuse through the polymer matrix of the biofilm and enough light absorption occurs by the PSs for a large number of microorganisms may be inactivated.[59-61]

Some studies evaluate the effect of aPDT by Laser. In this study, one LED was used instead of Laser device, and it has obvious economic. In addition, the lack of collimation and coherence of LED, which result in wider bands of emission (620–660 nm), providing light

emission throughout the entire absorption spectrum of the sensitizer, which may promote optimization of photodynamic processes.[18] Furthermore, Zanin et al[17] in 2005, demonstrated that the use of a HeNe Laser or a LED light in association with some photosensitizers showed the same antimicrobial effect over *S. mutans* biofilm.

Several studies have demonstrated the efficacy of a range of PSs in the killing of oral bacteria.[62-64] In dentistry, the most commonly PSs are based on phenothiazines derivative, such as methylene blue and toluidine blue.[52,53,65] Other PSs, such as Photogem® a hematoporphyrin derivated has been widely used to treat different kinds of cancers and in dentistry as antimicrobial agent during aPDT.[5] More recently, curcumin can also be considered a viable alternative to bacterial inactivation in the oral cavity. Studies, such as the Haukvik et al[65] and Dahl et al[50] showed that curcumin is used as an anticancer drug and has been shown to have antibacterial effects with toxic and phototoxicity effects over bacteria. Curcumin is an organic compound obtained from the yellow root of Curcuma longa (Zingiberaceae family) that is widely used as condiment, dye and medicine.[65]

Curcumin is shown to be a PS that is attached to the bacterial walls, drawing to itself the light at the time of irradiation with an essential antimicrobial action on oral bacteria. The concentration of curcumin used in this study was chosen based on another study that determined a safe concentration in terms of damage to the mucosa and discoloration of the teeth.[66]

The concentration of 1500 µg/ml is a low concentration and this fact can explain the results obtained. However, the great advantage to use curcumin is that it is a natural substance and harmless to the oral tissues.[40]

The statistical analysis indicated a significant decrease in *S. mutans* comparing with control. This finding confirms that the PSs used with light have antimicrobial activity against oral pathogens as seen in the work of Usacheva et al[66] and Williams et al.[52] Therefore, although the results obtained, which was carried out particularly on *S. mutans* are consistent with the findings of Fontana et al[61] and corroborate the results of O'Neill et al[57] and Zanin et al[17] aPDT can be indicated as a viable treatment for inhibition of microorganisms in dental biofilm.

Althought the statistical analysis showed a significant effect of aPDT, the results found in this study showed not a greater decrease in CFU for both PSs used. This result indicates a best performance for Photogem®. The results obtained with curcumin can be related with its lack of photochemical stability in solution which makes its potential to generate toxic oxygen decreased due to its rapid degradation.[67]

It is important to note that the PSs used selectively act on microorganisms, since only the areas irradiated by light produce reactive oxygen species (ROS)-singlet oxygen and free radicals-capable of eliminating microorganisms. The ROS act quickly, because they are very stable in the excited state by making these drugs less uptime on healthy oral tissues, thus avoiding undesirable side effects on them, being an advantage compared to antibacterial agents such as chlorhexidine, triclosan, fluoride or propolis.[67-69]

Antimicrobial photodynamic therapy can be an alternative therapy to prevent caries in dental plaque by decreasing the main etiological agent of dental caries, *S. mutans*, however, other photosensitizers and parameters need further investigation.

CONCLUSION

The results indicated that aPDT showed a low antibacterial performance over *S. mutans* biofilm, however, can be a promising alternative in the future to reduce bacterial activity in oral environment. Further studies exploring other PSs, their concentrations, light doses and their effect on biofilms may help to select appropriate light device and improve the molecular structure of PSs for better antibacterial activity.

These results showed that the association of curcumin/blue and Photogem®/red LED, although significant when compared with other groups (without aPDT), were not effective in reducing the viability of a large range of bacteria.

ACKNOWLEDGMENTS

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